

***Pestalotiopsis lijiangensis* sp. nov., a new endophytic fungus from Yunnan, China**

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ABSTRACT—A new endophytic fungus, *Pestalotiopsis lijiangensis* from leaves of *Castanopsis carlesii* var. *spinulosa*, is described and illustrated. The new species is similar to *P. montellica* and *P. jesteri* in having lateral appendages at the upper conidial septum, but *P. lijiangensis* differs from both *P. montellica* and *P. jesteri* by its wider conidia with deeply pigmented doliform median cells and additionally from *P. jesteri* by the production of apical conidial appendages. A phylogenetic analysis of the combined sequence data of internal transcribed spacer (ITS), partial β -tubulin (*tub*), and partial translation elongation factor1- α (*tef*) supports *P. lijiangensis* on an independent branch within *Pestalotiopsis*.

KEY WORDS—*Amphisphaeriales*, coelomycetes, molecular phylogenetics, *Pestalotiopsidaceae*, taxonomy

Introduction

Pestalotiopsis Steyaert is characterized by relatively fusiform conidia, each comprising a hyaline basal cell, three pigmented median cells, and a hyaline apical cell with two or more apical appendages (Steyaert 1949). The interspecific delineation of *Pestalotiopsis* is based on conidial morphology (Guba 1961, Nag Raj 1993), conidiogenesis (Sutton 1980), and teleomorph association (Barr 1975, 1990; Zhu & al. 1991; Metz & al. 2000). The use of molecular data in resolving *Pestalotiopsis* species has been reviewed by Hu & al. (2007), Tejesvi & al. (2007), Liu & al. (2010), and Maharachchikumbura & al. (2011). These studies suggest that multi-locus phylogenetic analysis is needed to resolve the

cryptic species in the genus. A combination of internal transcribed spacer (ITS), partial β -tubulin (TUB), and partial translation elongation factor1- α (TEF) gene data gave the best resolution when compared to single gene analysis (Maharachchikumbura & al. 2012). Based on these analyses, *Pestalotiopsis* sensu lato has been divided into three genera: *Pestalotiopsis* sensu stricto, *Neopestalotiopsis* Maharachch. & al., and *Pseudopestalotiopsis* Maharachch. & al., after morphology and sequence data (ITS, TUB, and TEF) were combined to identify new cryptic species (Maharachchikumbura & al. 2014).

Here, we isolated two endophytic fungal strains from a leaf of *Castanopsis carlesii* var. *spinulosa* W.C. Cheng & C.S. Chao (*Fagaceae*); both strains shared the character of lateral appendages at the upper conidial septum with *P. montellica* (Sacc. & Voglino) Tak. Kobay. and *P. jesteri* Strobel & al. (nom. inval.) but differed from those species by the size and pigmentation of their conidia. We therefore propose this endophytic fungus as a new species, *Pestalotiopsis lijiangensis*. Its phylogenetic position was inferred from the combined sequences of ITS, TUB, and TEF.

Materials & methods

Observation of morphological characters

Conidia were produced in a monoculture derived from a single conidium that was selected for morphological observation. Morphological characters for identification were measured and photographed under an Olympus Bx51 light microscope. These characters included conidial length and width, appendage length and number, and the pigmentation of the three median conidial cells. Microscopic examination performed 30 repetitions.

DNA extraction, amplification, sequencing

Total DNA was extracted from fresh cultures following the protocol of Guo & al. (2000). For nucleotide sequence comparisons, nrLSU, ITS, TUB and TEF genes were amplified using primer pairs LR0R/LR5 (Vilgalys & Hester 1990, Rehner & Samuels 1994), ITS5/ITS4 (White & al. 1990), T1/Bt-2b (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997), and EF1-728F/EF-2 (O'Donnell & al. 1998, Carbone & Kohn 1999), respectively. Amplification conditions for nrLSU, ITS and TEF followed Crous & al. (2012) and for TUB followed Lee & al. (2004). The PCR products were purified and edited by Zhongkexilin Biotechnology Co. Ltd. (Beijing, China). The new sequences were submitted to the GenBank database. Other sequences in this study were downloaded from GenBank (TABLE 1).

Phylogenetic analyses

The sequences were aligned with Clustal X1.81 (Thompson & al. 1997) and the results were adjusted manually where necessary to maximize alignment. All sites

TABLE 1. *Neopestalotiopsis* and *Pestalotiopsis* sequences used in the molecular analysis

SPECIES & VOUCHER	GENBANK ACCESSION No.		
	ITS	TUB	TEF
<i>N. saprophytica</i> MFLUCC12-0282	JX398982	JX399017	JX399048
<i>P. camelliae</i> CBS443.62	KM199336	KM199424	KM199512
<i>P. clavata</i> MFLUCC12-0268	JX398990	JX399025	JX39905
<i>P. cocculi</i> HHL-BZ	KM535703	KM573239	—
<i>P. colombiensis</i> CBS118553	KM199307	KM199421	KM199488
<i>P. disseminata</i> EC3A	EF055196	EF055233	—
<i>P. diversiseta</i> MFLUCC12-0287	JX399009	JX399040	JX399073
<i>P. gracilis</i> QQ-OZ	HM573281	HM535746	—
<i>P. hawaiiensis</i> CBS114491	KM199339	KM199428	KM199514
<i>P. intermedia</i> MFLUCC12-0259	JX398993	JX399028	JX399059
<i>P. jesteri</i> CBS109350	KM199380	KM199468	KM199554
" <i>P. jesteri</i> " MFLUCC12-0279	JX399012	JX399043	JX399076
<i>P. knightiae</i> CBS114138	KM199310	KM199411	KM199482
<i>P. lijiangensis</i> CFCC50738 [T]	KU860520	KU844184	KU844185
<i>P. lijiangensis</i> CFCC50739	MH8808834	MH880835	MH880836
<i>P. malayana</i> CBS102220	KM199306	KM199411	KM199482
<i>P. microspora</i> P20-02	HQ315843	—	—
<i>P. neglecta</i> QQ-TKZ	HM535758	HM573293	—
<i>P. olivacea</i> SY17A	EF055215	EF055251	—
<i>P. portugallica</i> CBS393.48	KM199335	KM199463	—
<i>P. rosea</i> MFLUCC12-0258	JX399005	JX399036	JX399069
<i>P. verruculosa</i> MFLUCC12-0274	JX398996	—	JX399061
<i>P. vismiae</i> HHL-DG	HM535704	HM573246	—

were treated as unordered and unweighted, and gaps were treated as missing data in the phylogenetic analyses. Firstly, new species in *Pestalotiopsis* sensu stricto, *Neopestalotiopsis*, and *Pseudopestalotiopsis* was estimated by the nrLSU sequence data. The result grouped *P. lijiangensis* in *Pestalotiopsis* sensu stricto (not shown). Analysis of a combined alignment dataset (ITS, TUB, and TEF) confirmed the relationship of the new species and other congeneric species in *Pestalotiopsis*. The alignment data was subsequently used for maximum parsimony (MP) analysis, in which a search for most parsimonious trees was conducted with the heuristic search algorithm with tree-bisection-reconnection (TBR) branch swapping in PAUP 4.0b10 (Swofford 2003). For each search, 1000 replicates of random stepwise sequence addition were performed, and all trees were saved per-replicate. The strength of the internal branches of the trees was tested with bootstrap analyses

using 1000 replications with the same search settings. For the Bayesian analysis, MrModeltest 3.7 with the Akaike information criterion was used to choose the substitution model for the separate dataset (Nylander & al. 2004), and the analysis was performed with MrBayes 3.1.2 (Huelsenbeck & Ronquist 2001, Ronquist & Huelsenbeck 2003). The detailed methods and parameters of MP and Bayesian analyses followed Maharachchikumbura & al. (2014). Bayesian posterior probabilities (PP) were obtained from the 70% majority rule consensus of the trees kept. Bootstrap percentages (BP) of more than 70 from 1000 replications are shown above the respective branches (FIG. 2). If >95% of the sampled trees were contained in a given clade, it was considered to be significantly supported by the data.

Taxonomy

Pestalotiopsis lijiangensis Y.K. Zhou & C.L. Hou, **sp. nov.**

FIG. 1

MYCOBANK MB 816081

Differs from *Pestalotiopsis montellica* by its wider conidia with deeply pigmented doliform median cells; and from *P. jesteri* by its wider conidia with deeply pigmented doliform median cells and by producing apical conidial appendages.

TYPE: China, Yunnan, Lijiang, alt. ca. 3800 m, endophytic in living leaves of *Castanopsis carlesii* var. *spinulosa*, 18 June 2014, Y.K. Zhou. **Holotype**, BJTC 1181; ex-type culture, CFCC50738; GenBank KU860520, KU844184, KU844185.

ETYMOLOGY: *lijiangensis* referring to the city where this species was obtained.

CONIDIOMATA pycnidia in culture on PDA, globose, scattered, semi-immersed, black, <100–300 µm diam; exuding globose, dark brown to black conidial masses. CONIDIOPHORES sparsely septate at base, branched or unbranched, subcylindrical, hyaline, smooth, <15 µm or reduced to conidiogenous cells. CONIDIOGENOUS CELLS discrete, lageniform to subcylindrical, hyaline, smooth, 10–25 × 2–5 µm ($x = 13.5 \times 3.7$ µm), proliferating 1–3 times percurrently. CONIDIA fusiform, 4-septate, 22–25 × 10–12 µm ($x = 23.8 \times 11.5$ µm), mean conidial length/width ratio = 2.96; bearing appendages; basal cell narrowly obconic with a truncate base bearing minute marginal frills, hyaline, smooth, 2–4 µm ($x = 3.4$ µm); three median cells doliform, brown, concolourous, walls verruculose and slightly constricted at the septa, together 14.5–18 µm ($x = 16$ µm), second cell from base 4.5–5.5 µm ($x = 5.3$ µm); third cell 4–5.1 µm ($x = 4.4$ µm); fourth cell 6–7.4 µm ($x = 6.3$ µm); apical cell obconic with an acute apex, hyaline, smooth, 2–3.5 µm ($x = 2.8$ µm). APPENDAGES tubular, unbranched, flexuous, more or less attenuated; apical appendage single, 3–8 µm ($x = 6.7$ µm); lateral appendages 2–4 (mostly 3), arising just above the septum separating the apical cell and subapical cell, unbranched 14–25 µm ($x = 18.6$ µm); basal appendage (occasionally absent) unbranched, attenuated, flexuous, centric 2–6 µm ($x = 4.8$ µm).

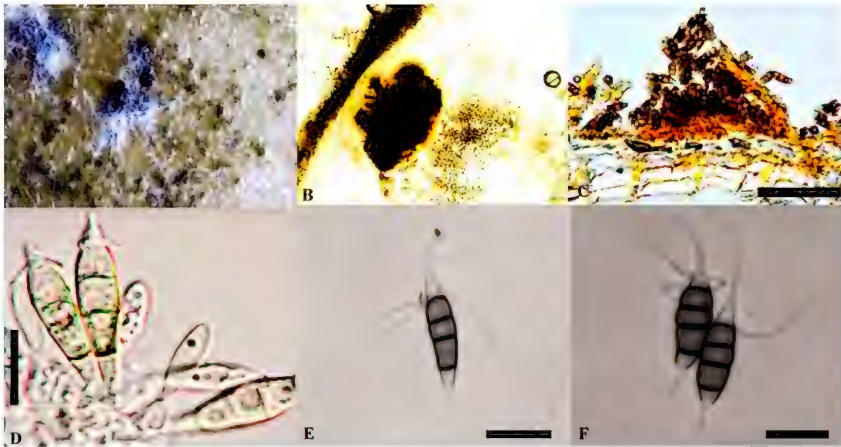


FIG. 1 *Pestalotiopsis lijiangensis* (CFCC50738): A. Conidiomata on PDA; B. Conidioma sporulating on PDA; C. Section of conidioma, split irregularly; D. Conidiophores and immature conidia; E, F. Conidia. Scale bars: C = 50 μ m; D = 30 μ m; E, F = 20 μ m.

ADDITIONAL MATERIAL EXAMINED: CHINA, YUNNAN, Lijiang, alt. ca. 3800 m, endophytic in living leaves of *Castanopsis carlesii* var. *spinulosa*, 18 June 2014, Y.K. Zhou (BJTC 1182, CFCC 50739; GenBank MH880834, MH880835, MH880836).

Phylogeny

To clarify species boundaries within *Pestalotiopsis*, a combined alignment of ITS, TUB, and TEF was created, containing 23 sequences (including the outgroup *Neopestalotiopsis saprophytica*) and 1519 characters. Of the 1519 characters (ITS = 552, TUB = 463, and TEF = 504), 890 were constant, 250 variable characters parsimony uninformative, and 379 characters parsimony informative. The MP analysis of sequences resulted in one most parsimonious tree with a length (TL) of 1738 steps, consistency index (CI) of 0.636, retention index (RI) of 0.708, homoplasy index (HI) of 0.604, and rescaled consistency index (RC) of 0.587. The Bayesian analysis resulted in a tree with the same topology and terminal clades as MP trees. *Pestalotiopsis* sensu stricto formed two well supported branches, and the new species *Pestalotiopsis lijiangensis* (CFCC 50738, CFCC 50739) was in a strongly supported independent clade (BP = 100, PP = 1) (FIG. 2).

Discussion

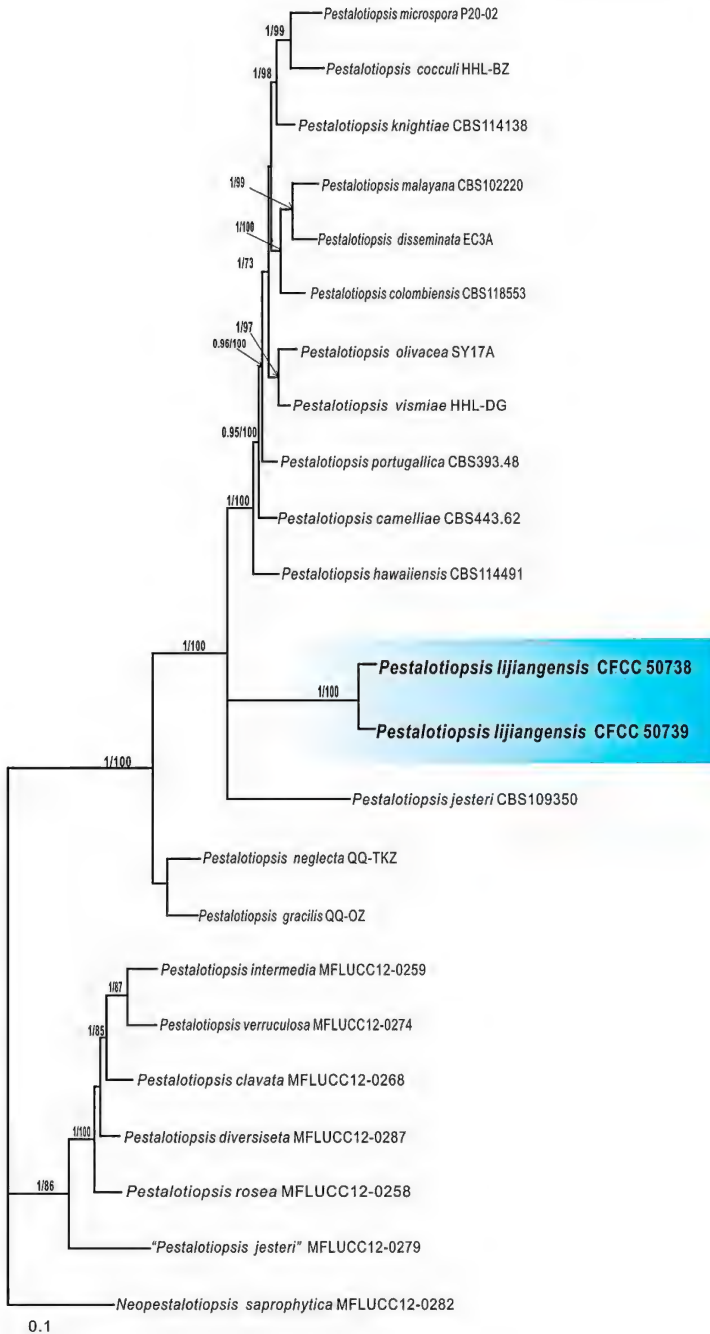
Conidial morphology is the most widely used taxonomic character for species in *Pestalotiopsis*, most species are identified by conidial size (Maharachchikumbura & al. 2011).

As can be seen from TABLE 2, the difference between *P. lijiangensis* and *P. montellica* is mainly the conidial length/width ratio, with the ratio of *P. montellica* almost 1.5 times that of *P. lijiangensis*. Furthermore, the lateral appendages of *P. montellica* are significantly shorter than those of *P. lijiangensis*. Although the conidial length/width ratios of *P. lijiangensis* and *P. jesteri* are similar, the three median cells are remarkably different: in *P. jesteri* the center cell is slightly smaller than the two others, while in *P. jesteri* the center cell is bigger (Strobel & al. 2000). Besides, the conidium of *P. jesteri* has no apical appendages, while *P. lijiangensis* has an unbranched apical appendage. Molecular analyses of the combined datasets cluster *P. lijiangensis* as an outlying species with high branch-length support. Unfortunately, no DNA sequence of *P. montellica* is available, so further research is needed to determine the relationship between *P. lijiangensis* and *P. montellica*.

TABLE 2. Morphological comparison of *Pestalotiopsis lijiangensis* and two similar species

CHARACTERS	SPECIES		
	<i>P. lijiangensis</i> (This paper)	<i>P. montellica</i> (Guba 1961)	<i>P. jesteri</i> (Strobel & al. 2000)
CONIDIUM MORPHOLOGY			
Length (μm)	18.5–27, <i>x</i> = 22.5	22–32, <i>x</i> = 26.5	19–23, <i>x</i> = 19
Width (μm)	6–9, <i>x</i> = 7.5	5–7, <i>x</i> = 5.7	5–7, <i>x</i> = 6
Mean length/width ratio	2.96	4.6	3.2
Median cell shape/color	Doliform/brown	Cylindrical/pale brown	Fusiform/pale brown
Lower median cell (μm)	4.5–6, <i>x</i> = 5.3	6–7.5, <i>x</i> = 6.7	4.8–5.2, <i>x</i> = 5
Center median cell (μm)	4–5.1, <i>x</i> = 4.4	3–5.5, <i>x</i> = 4.6	5.3–5.8, <i>x</i> = 5.5
Upper median cell (μm)	6–7.4, <i>x</i> = 6.3	4–6.5, <i>x</i> = 5.2	4.8–5.1, <i>x</i> = 4.9
APICAL APPENDAGES			
Number	1	1	0
Branching	unbranched	unbranched	—
Length (μm)	2–4, <i>x</i> = 3.4	5–7, <i>x</i> = 6	—
LATERAL APPENDAGES			
Number	(2–)3(–4)	(2–)3(–4)	3
Length (μm)	14–25, <i>x</i> = 18.6	4–12, <i>x</i> = 8	11–28, <i>x</i> = 23
BASAL APPENDAGES			
Number	(0–)1	0(–1)	1
Branching	Unbranched	Unbranched	Unbranched
Length (μm)	2–6, <i>x</i> = 4.8	2–6, <i>x</i> = 4	3–8, <i>x</i> = 4.5

FIG. 2 Phylogenetic tree derived from maximum parsimony analysis of the combined (ITS+TUB+TEF) sequences of *Pestalotiopsis* spp., with *Neopestalotiopsis. saprophytica* as outgroup. Branch support is shown as: Bayesian posterior probabilities >0.95 / bootstrap values >70%.



The two sequenced strains of *P. jesteri* appeared in different clades: CBS 109350 (a type strain from Papua New Guinea) was closely related to *P. lijiangensis*, while MFLUCC 12-0279 (from Yunnan, China) was only distantly related. The Yunnan strain was described with a conidial length/width ratio ≈ 4.1 and sequenced by Maharachchikumbura & al. (2012), who accepted it within *P. jesteri*. The Papua New Guinea strain, originally described by Strobel & al. (2000) and redescribed and sequenced by Maharachchikumbura & al. (2014), has a conidial length/width ratio ≈ 3.2 . The Yunnan strain was excluded from *P. jesteri* by Maharachchikumbura & al. (2014), who included only the original Papua New Guinea material in their circumscription of the species. Further study is needed to identify the misdetermined Yunnan strain MFLUCC 12-0279.

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Literature cited

- Barr ME. 1975. *Pestalospheeria*, a new genus in the *Amphisphaeriaceae*. *Mycologia* 67: 187–194. <https://doi.org/10.2307/3758246>
- Barr ME. 1990. Prodromus to nonlichenized pyrenomycetous members of class *Hymenoascomycetes*. *Mycotaxon* 39: 43–184.
- Carbone I, Kohn LM. 1999. A method for designing primer sets for speciation studies in filamentous *Ascomycetes*. *Mycologia* 91: 553–556. <https://doi.org/10.2307/3761358>
- Crous PW, Verkley GJM, Christensen M, Castaneda-Ruiz RF, Groenewald JZ. 2012. How important are conidial appendages? *Persoonia* 28: 126–137. <https://doi.org/10.3767/003158512X652624>
- Glass NL, Donaldson GC. 1995. Development of primer sets designed for use with the PCR to amplify conserved genes from filamentous ascomycetes. *Applied and Environmental Microbiology* 61: 1323–1330.
- Guba EF. 1961. Monograph of *Monochaetia* and *Pestalotia*. Harvard University Press, Cambridge.
- Guo LD, Hyde KD, Liew ECY. Identification of endophytic fungi from *Livistona chinensis* based on morphology and rDNA sequences. *New Phytologist* 147: 617–630. <https://doi.org/10.1046/j.1469-8137.2000.00716.x>
- Hu HL, Jeewon R, Zhou DQ, Zhou TX, Hyde KD. 2007. Phylogenetic diversity of endophytic *Pestalotiopsis* species in *Pinus armandii* and *Ribes* spp.: evidence from rDNA and β -tubulin gene phylogenies. *Fungal Diversity* 24: 1–22.
- Huelsenbeck JP, Ronquist F. 2001. MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* 17: 754–755. <https://doi.org/10.1093/bioinformatics/17.8.754>
- Lee S, Groenewald JZ, Crous PW. 2004. Phylogenetic reassessment of the coelomycete genus *Harknessia* and its teleomorph *Wuestneia* (*Diaporthales*), and the introduction of *Apharknessia* gen. nov. *Studies in Mycology* 50: 235–252.

- Liu AR, Chen SC, Wu SY, Xu T, Guo LD, Jeewon R, Wei JG. 2010. Cultural studies coupled with DNA based sequence analyses and its implication on pigmentation as a phylogenetic marker in *Pestalotiopsis* taxonomy. *Molecular Phylogenetics and Evolution* 57: 528–535. <https://doi.org/10.1016/j.ympev.2010.07.017>
- Maharachchikumbura SSN, Guo LD, Chukeatirote E, Bahkali AH, Hyde KD. 2011. *Pestalotiopsis*—morphology, phylogeny, biochemistry and diversity. *Fungal Diversity* 50: 167–187. <https://doi.org/10.1007/s13225-011-0125-x>
- Maharachchikumbura SSN, Guo LD, Cai L, Chukeatirote E, Wu WP, Sun X, Crous PW, Bhat DJ, McKenzie EHC, Bahkali AH. 2012. A multi-locus backbone tree for *Pestalotiopsis*, with a polyphasic characterization of 14 new species. *Fungal Diversity* 56: 95–129. <https://doi.org/10.1007/s13225-012-0198-1>
- Maharachchikumbura SSN, Hyde KD, Groenewald JZ, Xu J, Crous PW. 2014. *Pestalotiopsis* revisited. *Studies in Mycology* 79: 121–186. <https://doi.org/10.1016/j.simyco.2014.09.005>
- Metz AM, Haddad A, Worapong J, Long DM, Ford EJ, Hess WM, Strobel GA. 2000. Induction of the sexual stage of *Pestalotiopsis microspora*, a taxol-producing fungus. *Microbiology* 146: 2079–2089. <https://doi.org/10.1099/00221287-146-8-2079>
- Nag Raj TR. 1993. Coelomycetous anamorphs with appendage bearing conidia. *Mycologue Publications*, Waterloo, Ontario.
- Nylander JAA, Ronquist F, Huelsenbeck JP, Nieves-Aldrey JL. 2004. Bayesian phylogenetic analysis of combined data. *Systematic Biology* 53: 47–67. <https://doi.org/10.1080/10635150490264699>
- O'Donnell K, Cigelnik E. 1997. Two divergent intragenomic rDNA ITS2 types within a monophyletic lineage of the fungus *Fusarium* are nonorthologous. *Molecular Phylogenetics and Evolution* 7: 103–116. <https://doi.org/10.1006/mpev.1996.0376>
- O'Donnell K, Cigelnik E, Nirenberg HI. 1998. Molecular systematics and phylogeography of the *Gibberella fujikuroi* species complex. *Mycologia* 90: 465–493. <https://doi.org/10.2307/3761407>
- Ronquist F, Huelsenbeck JP. 2003. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 19: 1572–1574. <https://doi.org/10.1093/bioinformatics/btg180>
- Rehner SA, Samuels GJ. 1994. Taxonomy and phylogeny of *Gliocladium* analysed from nuclear large subunit ribosomal DNA sequences. *Mycological Research* 98: 625–634. [https://doi.org/10.1016/S0953-7562\(09\)80409-7](https://doi.org/10.1016/S0953-7562(09)80409-7)
- Steyaert RL. 1949. Contribution à l'étude monographique de *Pestalotia* de Not. et *Monochaetia* Sacc. (*Truncatella* gen. nov. et *Pestalotiopsis* gen. nov.). *Bulletin du Jardin Botanique de l'État, Bruxelles* 19: 285–347. <https://doi.org/10.2307/3666710>
- Strobel G, Li JY, Ford E, Worapong J, Baird GI, Hess WM. 2000. *Pestalotiopsis jesteri*, sp. nov. an endophyte from *Fragaria bodenii*, a common plant in the southern highlands of Papua New Guinea. *Mycotaxon* 76: 257–266.
- Sutton BC. 1980. The coelomycetes. Fungi imperfecti with pycnidia, acervuli and stromata. Commonwealth Mycological Institute, Kew, Surrey, UK.
- Swofford DL. 2003. PAUP*, Phylogenetic analysis using parsimony (*and other methods). Version 4.0. Sinauer Associates, Sunderland, Massachusetts.
- Tejesvi MV, Kini KR, Prakash HS, Subbiah V, Shetty HS. 2007. Genetic diversity and antifungal activity of species of *Pestalotiopsis* isolated as endophytes from medicinal plants. *Fungal Diversity* 38: 37–54.
- Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* 25: 4876–4882. <https://doi.org/10.1093/nar/25.24.4876>

- Vilgalys R, Hester M. 1990. Rapid genetic identification and mapping of enzymatically amplified ribosomal DNA from several *Cryptococcus* species. *Journal of Bacteriology* 172: 4238–4246. <https://doi.org/10.1128/jb.172.8.4238-4246.1990>
- White TJ, Bruns T, Lee S, Taylor J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. 325–322, in: MA Innis & al. (eds). *PCR protocols: a guide to methods and applications*. Academic Press, San Diego. <https://doi.org/10.1016/B978-0-12-372180-8.50042-1>
- Zhu PL, Ge QX, Xu T. 1991. Seven new combinations of *Pestalotiopsis* from China. *Acta Mycologica Sinica* 10: 273–279.